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The overall objective of this study was to establish the pharmacology of the ionic channel of the peripheral nicotinic acetylcholine (ACh) receptor and to understand the mechanism by which this receptor controls the selective translocation of cations across membranes. The combination of electrophysiological techniques with biochemical ones helped us to understand the molecular properties of this ACh-receptor/channel and its interactions with drugs and toxins and also determine the cases where it may act as a secondary target for the action of a variety of drugs including anticholinesterases.	

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Electrophysiological techniques were used to analyze the characteristics of endplate currents and channel properties of mammalian and frog skeletal muscles, while biochemical techniques were used to study the molecular characteristics and drug specificities of the ACh-receptor/channel of the electric organ of the electric ray, Torpedo sp.

The data obtained suggest that the ACh-receptor/channel molecule carries two kinds of binding sites: receptor sites that bind ACh and  $\alpha$ -bungarotoxin in a voltage-independent and almost temperature-independent manner, and channel sites that bind a variety of drugs in a voltage- and temperature-dependent manner, and are more sensitive than the receptor sites to treatment by the detergent Triton X-100 and sulfhydryl reducing and alkylating agents. The drugs that bind to the channel sites include perhydrohistrionicotoxin, the antiviral and antiparkinson drug amantadine, local anesthetics (e.g., piperocaine), opiate antagonists (e.g., naltrexone) and agonists (e.g., levallorphan and cyclazocine), the antimalarial quinacrine, antibiotics (e.g., polymyxin, gentamicin and aminoglycosides), tricyclic antidepressants such as imipramine, the general anesthetic and hallucinogen phencyclidine, as well as the receptor site antagonists decamethonium and curare, anticholinesterase quaternary ammonium compounds and aliphatic alcohols. The molecular diversity of these drugs demonstrates the relative nonspecificity of the channel sites, while the high affinities that many drugs have ( $K_i < 1$  M) suggest that they may be secondary targets for many drugs. Two of the toxins studied (anatoxin and nereisotoxin) did not bind to the channel sites, but rather to the receptor sites, acting as agonists and antagonists, respectively. There are different ionic channel sites reachable either from only the outside of the membrane (e.g., atropine methyl bromide and tetraethylammonium) or from both outside and inside (e.g., PCP methiodide and piperocaine methiodide). In addition, PCP and perhydrohistrionicotoxin bind competitively to the same channel sites, while imipramine appears to bind non-competitively to different and fewer number of channel sites.

The ACh-receptor/channel molecule undergoes different ligand-induced conformations, which we could detect by monitoring the apparent rates of binding of radiolabeled drugs to the channel sites. These are the resting, agonist-induced, antagonist-induced and desensitized conformations. Several of the channel drugs bind to open as well as closed channel conformations, and some such as imipramine bind preferentially to an intermediate activated but nonconducting conformation. The allosteric effect of receptor site occupation on channel site affinity is inhibited by  $\text{Ca}^{2+}$ , while F<sup>-</sup> reverses the  $\text{Ca}^{2+}$  effect.

It is evident that the pharmacology of the ionic channel sites of the nicotinic ACh-receptor is very different from, and less specific than, that of the receptor sites. The high affinity the channel sites have for many drugs makes them viable, though secondary, targets for their actions. This has importance particularly in compromised neuromuscular transmission such as in certain patients (e.g., with myasthenia gravis) and in cases of poisoning by anticholinesterases.

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THE ACETYLCHOLINE RECEPTOR AND ITS IONIC CHANNEL  
AS TARGETS FOR DRUGS AND TOXINS

FINAL REPORT

MOHYEE E. ELDEFRAWI AND EDSON X. ALBUQUERQUE

DECEMBER 10, 1981

U.S. ARMY RESEARCH OFFICE

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## 2. Foreword

This work was carried out under U.S. Army Research Office grant DAAG 29-78G\_0203. In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care,": as promulgated by the Committee on the Guide for Laboratory Animals, Resources, National Academy of Sciences-National Research Council.

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## 5. Body of Report

### 5a. Statement of the Problem Studied

The overall objective of this study was to establish the pharmacology of the ionic channel of the nicotinic acetylcholine (ACh)-receptor in muscle and fish electric organ, and to understand the mechanism by which this ACh-receptor controls the selective translocation of cations across membranes. The combination of electrophysiological techniques with biochemical ones helped us understand the molecular properties of this ACh-receptor channel and its interactions with drugs and toxins and also determine the cases where it may act as a secondary target for the actions of a variety of drugs including anticholinesterases.

### 5b. Summary of the Most Important Results

#### 5b.i. Results of Biochemical Experiments:

Earlier, electrophysiological studies on skeletal muscle had suggested that the ACh-receptor/channel complex had sites that bound acetylcholine (ACh) and ( $\alpha$ -BGT) (identified as receptor sites) and sites that bound local anesthetics and  $H_{12}$ -HTX (Albuquerque et al., 1973, 1974; Krodel et al., 1979; Kato and Changeux, 1976). Our studies on the interactions of [ $^3H$ ]perhydropyridylhistricotxin ([ $^3H$ ]H $_{12}$ -HTX) with the electric organ of the electric ray, *Torpedo* sp. suggested that the detected binding was indeed to the ionic channel sites (Eldefrawi et al., 1977, 1978) as was also later reported by others (Elliott and Raftery, 1977). To confirm this conclusion we studied the correlation between the ability of drugs to displace [ $^3H$ ]H $_{12}$ -HTX binding to *Torpedo* membranes and their ability to block neuromuscular transmission. We found that drugs which selectively blocked neuromuscular transmission in a voltage-dependent and sometimes time-dependent manner also blocked binding of [ $^3H$ ]H $_{12}$ -HTX to *Torpedo* membranes. Among those were drugs that had very low affinity ( $K_d > mM$ ) for the receptor site (e.g., amantadine (Tsai, et al., 1978), phencyclidine (PCP) (Albuquerque et al., 1980a) and imipramine (Eldefrawi et al., 1981). There were other drugs which blocked neuromuscular transmission in a voltage- and time-dependent manner and blocked binding of H $_{12}$ -HTX to *Torpedo* membranes, but they also interfered with the binding of [ $^3H$ ]acetylcholine ([ $^3H$ ]ACh) and [ $^{125}I$ ]-bungarotoxin ([ $^{125}I$ ]-BGT) to the receptor sites. Thus, these drugs interact with both the receptor as well as the channel sites. Among the drugs which belong to this second group are the antimalarial drug quinaquine (Adams and Feltz, 1980; Tsai et al., 1979), the local anesthetic piperocaine (Tiedt et al., 1979) and the ganglionic blocker tetraethylammonium (Adler et al., 1979). The excellent correlation obtained between the potencies of several histricotxin analogs in reducing the amplitude of endplate currents (EPC) and their potencies in displacing [ $^3H$ ]H $_{12}$ -HTX binding (Eldefrawi and Eldefrawi, 1979) was strong evidence that H $_{12}$ -HTX was indeed interacting with the ionic channel site.

It was clear from these initial studies that although the biochemical and electrophysiological studies used two tissues which represented distant species phylogenetically, their two receptor/channel systems were similar. The excellent correlation found between the effect of H $_{12}$ -HTX in blocking receptor-regulated influx of  $^{22}Na^+$  in *Torpedo* microsacs (Eldefrawi et al., 1980a) and blockade of the ACh sensitivity of the extrajunctional receptors of

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denervated rat soleus muscle (Iapa et al., 1975) suggested that measurements of specific  $^{22}\text{Na}$  flux represented receptor-regulated flux.

In the above studies, the assay used for detection of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding to Torpedo membranes was measuring equilibrium binding by dialysis or centrifugation. A new dimension in the biochemical investigations started with our development of a rapid filter assay that monitored time-dependent events. Exposure of the Torpedo membranes to the channel probe  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  then filtering rapidly on Whatman GF/B filters (pretreated with organosilane to reduce nonspecific binding) allowed monitoring of the apparent rate of binding and the effect of receptor stimulation on the binding of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$ . The binding of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  was very slow, reaching equilibrium in about 90-120 min, but in presence of agonist maximal binding was reached very fast (Eldefrawi et al., 1980a; Aronstam et al., 1981). Also, the affinity of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding was increased in the presence of agonist from  $K_d$  of 0.1  $\mu\text{M}$  to 0.082  $\mu\text{M}$ . Thus, the initial rate of binding (i.e., in 30 sec) was increased by carbamylcholine (carb) up to a hundredfold in a dose-dependent manner up to about 10  $\mu\text{M}$  carbamylcholine (carb), then decreased at higher concentrations possibly reflecting receptor desensitization. Preincubation with lower concentrations carb also reduced the carb-induced increased initial rate of binding in a time-dependent manner. Other receptor agonists (e.g., ACh, nicotine, succinylcholine and decamethonium also stimulated the initial rate of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding in a concentration-dependent manner (Aronstam et al., 1981) corresponding to their potencies in depolarizing postsynaptic membranes of Torpedo electric organ (Moreau and Changeux, 1976). This increase in  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding corresponds to its increased potency of EPC's subsequent to activation of the ACh-receptor in skeletal muscles (Nagukawa and Albuquerque, 1978). These findings suggested that the increase in  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding by agonist reflected binding to an activated conformation of the ionic channel and proved the coupling between the agonist and channel binding sites (Eldefrawi and Eldefrawi, 1980b). Binding of receptor antagonists (curare, gallamine) also increased the initial rate of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding, which suggested a possible fourth conformation that could be detected in addition to the resting, active and desensitized ones. Receptor activation by agonists also affected the affinity of drugs to sites on the ionic channel; the affinities of some increased, others decreased, while a third group was unaltered (Aronstam et al., 1980a).

Because of the limited supply of  $[^3\text{H}]\text{H}_{12}\text{-HTX}$ , it was important to search for other radiolabeled probes for the ionic channel of the ACh-receptor. Recently we discovered that the general anesthetic and psychotomimetic PCP interacted with and inhibited several targets in the nervous system and muscle, including the electrogenic  $\text{K}^+$  channel and the ionic channel of the nicotinic receptor (Albuquerque et al., 1980a, b; Tsai et al., 1980; Aronstam et al., 1980b). PCP did not inhibit  $[^{125}\text{I}]\alpha\text{-BGT}$  or  $[^3\text{H}]\text{ACh}$  binding to Torpedo membranes, as reported by Weinstein et al. (1973), but it inhibited  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  binding. The results of biophysical and biochemical experiments indicated similarities between  $\text{H}_{12}\text{-HTX}$  and PCP. They both had voltage-dependent and time-dependent effects on EPC of frog endplates causing nonlinearity in the current-voltage relationship and accelerating the EPC decay phase by reacting with the closed as well as the open conformations of the receptor/channel molecule, with a higher affinity for the open conformation. The commercially available  $[^3\text{H}]\text{PCP}$  bound to a finite number of sites in Torpedo membranes equal to those that bound  $[^3\text{H}]\text{H}_{12}\text{-HTX}$  and did not

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inhibit [ $^3\text{H}$ ]ACh or [ $^{125}\text{I}$ ] $\alpha$ -BGT binding to the receptor sites. Their binding was competitive, had the same drug specificity, and the initial rate of [ $^3\text{H}$ ]PCP binding was also increased greatly in presence of carb, suggesting that this binding was to the ionic channel of the ACh-receptor and [ $^3\text{H}$ ]PCP was a good substitute for [ $^3\text{H}$ ]H<sub>12</sub>-HTX as a probe for this channel in Torpedo membranes (Eldefrawi et al., 1980b). However, in brain membranes, PCP binds to many sites, and identification of its specific binding to the nicotinic receptor's ionic channel faces many obstacles. A major difference between the two channel probes [ $^3\text{H}$ ]H<sub>12</sub>-HTX and [ $^3\text{H}$ ]PCP was that in using the latter, two affinities for binding to Torpedo membranes could be detected in absence of receptor agonist ( $K_d$  0.1 and 50  $\mu\text{M}$ ), but only one with a  $K_d$  of 3.1  $\mu\text{M}$  in its presence). The affinities of various channel drugs obtained by inhibition of binding of [ $^3\text{H}$ ]PCP and [ $^3\text{H}$ ]H<sub>12</sub>-HTX to Torpedo membranes were different, with correlation of 0.52 and 0.82 in the absence and presence of a receptor agonist, respectively. It suggested that there were some differences in their binding sites on the ionic channel, which were more apparent in the resting conformation (Eldefrawi et al., 1980b).

Another probe that we discovered for the ionic channel sites is the tricyclic antidepressant imipramine (IMIP). It selectively blocked the binding of [ $^3\text{H}$ ]H<sub>12</sub>-HTX, but not [ $^3\text{H}$ ]ACh or [ $^3\text{H}$ ] $\alpha$ -BGT to Torpedo membranes. On the frog endplate the drugs had voltage-dependent inhibition of neuromuscular transmission but did not alter the kinetics of the time constant of decay. The data suggested that IMIP favored binding to the activated but nonconducting conformation of the ionic channel and also causes partial block of the channel in its open conformation (Eldefrawi et al., 1981). Also, IMIP bound to sites on the ionic channel that were different from the ones that bound [ $^3\text{H}$ ]H<sub>12</sub>-HTX or [ $^3\text{H}$ ]PCP, since IMIP inhibition of this binding was noncompetitive, and the total number of [ $^3\text{H}$ ]IMIP binding sites was about 0.3 sites that bound [ $^3\text{H}$ ]H<sub>12</sub>-HTX or [ $^3\text{H}$ ]PCP. Several other tricyclic antidepressants inhibited the binding of [ $^3\text{H}$ ]IMIP to Torpedo membranes (Aronstam, 1981; Shaker et al., 1981), and their order of potency was similar to that reported for their effect on [ $^3\text{H}$ ]IMIP binding to mammalian brain (Raisman et al., 1980). However, the concentration of these tricyclic antidepressants are at least 10-fold higher than their therapeutic concentrations.

Our investigation of the interactions of two depolarizing blockers of the ACh-receptor/channel (decamethonium and succinylcholine) revealed that these drugs had different mechanisms for blocking the receptor function. Decamethonium, which was suggested to depolarize the receptor at low concentration and block the ionic channel at higher concentrations (Adams and Sakmann, 1978), increased the initial rate of [ $^3\text{H}$ ]H<sub>12</sub>-HTX binding to Torpedo membranes at low concentrations and blocked binding at higher concentrations. To confirm that the reduction in binding was due to channel blockade rather than desensitization, the receptor sites were first irreversibly occupied during the experiment with  $\alpha$ -BGT, then the binding of [ $^3\text{H}$ ]H<sub>12</sub>-HTX (whose binding to the resting conformation of the ionic channel was unaffected by  $\alpha$ -BGT) was studied in presence of decamethonium and succinylcholine. As shown, only decamethonium was capable of blocking binding of [ $^3\text{H}$ ]H<sub>12</sub>-HTX to the ionic channel site. On the other hand, succinylcholine appears to block neuromuscular transmission through quick desensitization of the system.

If an agent produced effective and fast desensitization it would be quite toxic. Anatoxin-a is such an agent, whose common name is the quick death factor. This bicyclic amine, the exotoxin isolated from a filamentous fresh water blue algae Anabaena sp., acted as an agonist on the nicotinic ACh-receptor in electrophysiologic experiments (Spivak and Albuquerque, 1980). It inhibited [ $^3$ H]ACh and [ $^{125}$ I] $\alpha$ -BGT binding to Torpedo membranes but not [ $^3$ H]H<sub>12</sub>-HTX binding (Spivak et al., 1979). It also inhibited binding of [ $^3$ H]QNB to muscarinic receptors with similarly high affinities. Thus, acted similar to ACh on both nicotinic and muscarinic synapses and did not interact with the nicotinic ionic channel sites.

A series of quaternary ammonium anticholinesterases were studied for their interaction with the ACh-receptor and ACh-esterase. Some of these drugs had equal or better affinity for the receptor than the esterase (Table 2). There was no correlation between their potencies in inhibiting ACh-esterase and theirs on the ACh-receptor, suggesting that their binding sites in the two proteins are very different. All activated the receptor though to a lesser degree than carbamylcholine as shown by their stimulation of receptor-regulated  $^{22}$ Na<sup>+</sup> flux in Torpedo membranes. In presence of carbamylcholine, these drugs inhibited receptor response possibly because they occupied the site and produced little activation but prevented accessibility of the full agonist.

The effect of the carbamate anticholinesterase neostigmine on the binding of receptor and channel ligands was studied in an effort to explain the anomalous effects of this drug on neuromuscular transmission discussed by Stevens in a recent review (Stevens, 1978). We found that neostigmine bound to the receptor and inhibited binding of [ $^3$ H]ACh to Torpedo receptors, but its affinity was at least two orders of magnitude lower than d-tubocurarine and carbamylcholine. However, neostigmine increased the initial rate of binding of [ $^3$ H]H<sub>12</sub>-HTX like agonists but to a much lower extent when compared to carbamylcholine. It also inhibited the carbamylcholine-stimulated binding as antagonists would. These results suggested that neostigmine could bind to the receptor and activate it like a partial rather than a full agonist.

Narcotic analgesic antagonists also interacted with the ACh-receptor/channel. Naltrexone, a pure opiate antagonist, shortened the time constant of decay of both EPC and MEPC of frog sartorius muscle and caused nonlinearity in the current/voltage relationship (Schofield et al., unpublished results). Naltrexone inhibited noncompetitively the receptor-regulated [ $^3$ H]H<sub>12</sub>-HTX binding, but the affinity was fairly low ( $K_i$  = 200  $\mu$ M). At these concentrations naltrexone had no effect on the binding of [ $^3$ H]ACh to its receptor. Using [ $^3$ H]PCP as the channel probe we found that opiate agonists like nalorphine and morphine had very little effect on the carbamylcholine-stimulated [ $^3$ H]PCP binding, whereas the psychotomimetic antagonists levallorphan, cyclazocine and SKF 10,047 were quite effective. Levallorphan, a mixed antagonist of morphine, also had a similar depressant action on both the EPC and peak amplitude and time course of the EPC's although it was more potent.

Alcohols block peripheral nerves by interfering with both Na<sup>+</sup> and K<sup>+</sup> conductances, but they also affect receptor/channel interactions in a unique manner. Like receptor agonists, aliphatic alcohols at low concentrations increased the rates of binding of three channel probes, while at high

concentrations they decreased them. However, unlike agonists, they interfered with the carb-induced increased rate of binding of channel probes, and at the endplate, they did not depolarize the postsynaptic membrane, but prolonged the decay phase of MEPP (Gage et al., 1975). This suggests that they affect the receptor/channel conformation by a mechanism that is different from the receptor and channel drugs discussed above.

Nereistoxin (NTX), a toxin isolated from the marine segmented worm Lumbriconereis heteropoda reduced the amplitudes of the EPP and EPC in frog sartorius and rat diaphragm muscles as well as the extrajunctional ACh sensitivity of denervated rat soleus muscle, and also inhibited binding of [ $^3$ H]ACh and [ $^{125}$ I] $\alpha$ -BGT to Torpedo ACh-receptor sites (Eldefrawi et al., 1980c). In addition, NTX caused initial postsynaptic depolarization and potentiation of the indirectly elicited twitch tension. Since NTX did not inhibit binding of [ $^3$ H]H $_{12}$ -HTX to Torpedo membranes and did not alter the linearity of the current voltage relationship, nor the time course of EPC, we suggested that its inhibition of neuromuscular transmission was due to its inhibition of the ACh-receptor sites and not the ionic channel sites. Also, NTX acted as a partial agonist since it could activate the ACh-receptor-regulated  $^{22}$ Na $^+$  flux in Torpedo microsacs although its major action was that of an antagonist since it inhibited the carbamylcholine-activated  $^{22}$ Na influx, thus acting as a partial agonist or antagonist of the ACh-receptor.

At the beginning of this project we had suspected that receptor and channel may be independent molecular entities which are coupled in the membrane (Eldefrawi et al., 1977, 1978). A similar viewpoint was expressed by others (Sobel et al., 1977). Using equilibrium binding assays we detected little or no [ $^3$ H]H $_{12}$ -HTX binding to the purified ACh-receptor protein even after using cholate instead of Triton X-100. Furthermore, while all of the [ $^3$ H]ACh binding sites were adsorbed by Naja  $\alpha$ -toxin affinity gel from cholate extracts, only half of the [ $^3$ H]H $_{12}$ -HTX was adsorbed. The remaining [ $^3$ H]H $_{12}$ -HTX binding had drug specificity similar to the sites in the membrane (Eldefrawi et al., 1978). Recently, however, after adapting the filter assay for [ $^3$ H]H $_{12}$ -HTX binding to soluble receptor preparations, we discovered that the receptor-stimulated [ $^3$ H]H $_{12}$ -HTX binding was totally removed by the affinity gel. This, plus the finding that axonal K $^+$  channels bind many of the drugs that affect the ACh-receptor/channel molecule (e.g., PCP and tetraethylammonium), argue in favor of the receptor and channel sites being on the same molecule. This is supported by the recent successful reconstitution of the purified protein which exhibits proper receptor function. Thus, the fraction of [ $^3$ H]H $_{12}$ -HTX which was not removed by the affinity gel in previous experiments could be K $^+$  channels rather than receptor/channels. We have modified our purifications protocols to obtain a final product which retains its ability to bind [ $^3$ H]H $_{12}$ -HTX and show receptor/channel coupling.

The effect of various treatments on the binding of receptor as well as channel ligands to Torpedo membranes has given us important information on their molecular properties. We found that channel site binding was much more temperature dependent than the receptor site (Eldefrawi and Eldefrawi, 1980b), suggesting that the H $_{12}$ -HTX channel sites are deeper within the membrane matrix. However, treatment of Torpedo membrane with trypsin inhibited [ $^3$ H]H $_{12}$ -HTX in absence as well as presence of carbamylcholine. Assuming that the microsacs used contain ACh-receptor facing the outside (Hartig and Raftery, 1979), the data suggest that most or all these channel sites are

accessible to trypsin from outside the cell. pH also had a differential effect on the binding of receptor and channel ligands only if the drug was ionizable. [ $^3\text{H}$ ]H<sub>12</sub>-HTX, which has a pK<sub>a</sub> of 9, has a pH profile similar to that of ACh. Nicotine, which binds mainly in the protonated form, has a pH profile different from that of H<sub>12</sub>-HTX binding. The results suggest different ionizable groups at the receptor and channel sites. The differential effect of sulfhydryl reagents such as DTT, PCMB, mercaptoethanol and HgCl<sub>2</sub> on the binding of [ $^3\text{H}$ ]ACh and [ $^3\text{H}$ ]H<sub>12</sub>-HTX suggest that sulfhydryl function is important for both sites. The effect of detergents is most interesting. Triton X-100, which has been used so successfully to solubilize the receptor, inhibits binding of [ $^3\text{H}$ ]H<sub>12</sub>-HTX effectively. Cholic acid derivatives are much less inhibitory.

Our earlier studies showed that Ca<sup>2+</sup> was closely associated with the receptor protein (Eldefrawi et al., 1975), and that binding of receptor agonists but not antagonists caused loss of Ca<sup>2+</sup> from the receptor (Rubsamen et al., 1976a, b; Eldefrawi et al., 1977). Our recent studies (unpublished) show that Ca<sup>2+</sup> depressed significantly the receptor-stimulated increase in the initial rate of binding of [ $^3\text{H}$ ]H<sub>12</sub>-HTX. This effect is similar to the effect of desensitization on the binding of [ $^3\text{H}$ ]H<sub>12</sub>-HTX, and Ca<sup>2+</sup> is known to promote endplate desensitization (Miledi and Parker, 1980). Accordingly, we tested the effect of agents such as fluoride ions (F<sup>-</sup>), which have been described to delay desensitization (Kaibara et al., 1978), on the effect of Ca<sup>2+</sup> on binding. Our results showed clearly that F<sup>-</sup>, which increased ACh-induced conductances (Eldefrawi and Albuquerque, unpublished), reversed the effect of Ca<sup>2+</sup> on the receptor-stimulated [ $^3\text{H}$ ]H<sub>12</sub>-HTX binding (Fig. 18). Other anions (Cl<sup>-</sup>, I<sup>-</sup> or Br<sup>-</sup>) did not produce this effect. Ethyl alcohol, which increased ACh sensitivity (Gage et al., 1975), antagonized the Ca<sup>2+</sup> effect at the same concentrations. However, while it is easy to think of F<sup>-</sup> as forming insoluble complexes with Ca<sup>2+</sup> we are not certain how ethanol antagonizes the Ca<sup>2+</sup> effect.

#### 5b.ii. Results of the Electrophysiological Experiments:

During the tenure of this grant and with the objective of fulfilling its aims and objectives, the understanding of the activation and inactivation of the nicotinic ACh receptor of the neuromuscular junction has been a subject of major concern. By understanding this process and the different species that are generated including the conformational change which occurs in the receptor molecule upon reaction with the agonist, and the sequence of events which culminates with opening of the ionic channel, one would be able to study the dynamic properties of the molecule and its ability to react at any given state with the drugs and toxins. The action of the large concentrations of the agonists, phosphorylated compounds, certain toxins, and antagonists of the ionic channel are of key importance. Indeed, a large number of known neuromuscular blocking agents were assumed until recently to compete with ACh for a common recognition site. This view was dismissed as too simple when agents such as histrionicotoxin (HTX) were discovered and became known as potent endplate blockers with negligible affinity for the ACh recognition site. Although HTX does not interact with the ACh recognition site, the agent is able to create a situation in which even large quantities of agonist at synaptic cleft are not able to produce marked changes in synaptic function because HTX is able to specifically block the mechanisms by which translocation occurs between the receptor and the contractile elements. In

other synapses HTX and analogs prevent the final stage of activation of the ionic channel produced by the agonist. Therefore, in compliance with the initial proposal, we have used several derivatives of HTX which appear to affect significantly the ionic channel of the ACh receptor and thus provide important information regarding the different binding sites which control the peak amplitude and decay time constant of the endplate current (EPC). These experiments have added immensely to our knowledge of the physiology and molecular pharmacology of the ionic channel of the nicotinic ACh receptor.

Natural and semisynthetic analogs of the saturated alkaloid histrionicotoxin (HTX; (2pR, 6S, 7OS, 8aS)-7-(cis-1-buten-3-ynyl)-8-hydroxy-2-(cis-2-penten-4-ynyl)-1-azabicyclo [5.5] undecane) are:  $H_4$ -iso,  $H_8$ - and  $H_{12}$ -HTX. Both HTX and  $H_{12}$ -HTX have been examined for their ability to block K conductance and to interact with the ionic channel of the ACh receptor. Both HTX and  $H_{12}$ -HTX decreased the endplate current peak amplitude in a voltage- and time-dependent fashion giving rise to a hysteretic current-voltage relationship such as that shown for HTX (Spivak and Albuquerque, 1981). For example, at -90 mV, with 10, 20, 30 and 40  $\mu$ M HTX, the peak EPC amplitude was reduced by 56, 86, 90 and 92% while the corresponding reduction in  $\tau$  was 38, 47, 47 and 49%. With 10, 20, 30 and 40  $\mu$ M  $H_{12}$ -HTX, the peak EPC amplitude was reduced by 61, 72, 84 and 89%, while the corresponding reduction in  $\tau$  was 36, 40, 43 and 44%, respectively. The depression of peak EPC amplitude is concentration dependent up to at least 40  $\mu$ M of the toxins. While the time- and voltage-dependence induced by various concentrations of HTX gave rise to the curvature and hysteresis which appears to be concentration-related, the decay time constant ( $\tau$ ) reached equilibrium and saturation at about 10 minutes. This clearly demonstrated that the toxin depressed the peak amplitude independently of  $\tau$  in the time required to reach equilibrium (Spivak and Albuquerque, 1981). The  $\tau_{EPC}$  reached its equilibrium value in about 10 minutes while the peak amplitude is still declining at 30 minutes. The  $\tau_{EPC}$  plots can also provide evidence for closed channel block.  $H_{12}$ -HTX, for example, yielded a  $1/\tau$  versus drug concentration curve that showed distinct saturation (Spivak and Albuquerque, 1981, unpublished results). All of the decays of the EPC were single exponential functions. Since there is no way that one could account for the results by an open blocking scheme, we propose that HTX and most of its analogs bind to some channel sites that permit activation but with different kinetics. At 4  $\mu$ M  $H_4$ -iso-HTX and 25  $\mu$ M  $H_8$ -HTX, peak EPC amplitude was reduced by 76 and 65% and  $\tau$  was shortened by 31 and 34%, respectively, values similar to those seen with HTX and  $H_{12}$ -HTX.  $H_4$ -iso-HTX appears to be the most potent compound. Under control conditions, the amplitude of the EPC increased initially during tetanic stimulation (25 Hz for 1 sec) and then returned to control level at the end of the train. Within 10 sec the tetanic train could be repeated 2-3 times without any difference. In the presence of HTX (35  $\mu$ M) or  $H_{12}$ -HTX (30  $\mu$ M), there was a slight potentiation of the first 2-3 EPCs and then a rapid rundown of the EPC amplitude. At the end of the train the EPC amplitude was depressed by 57%. With 20 sec rest intervals (or less) between tetanic stimulation, subsequent trains of EPCs could be elicited in which the initial EPC in the second and third trains were 65 and 57% of the initial EPC in the first train, respectively. Although the peak amplitude of the EPC decreased significantly during the trains of stimuli,  $\tau$  was unaltered during the entire tetanus (first, second or third trains). At 35  $\mu$ M HTX, the mean value of  $\tau$  of the first, fifth and last EPC during any tetanus was 65, 67 and 69% of control, respectively. With  $H_{12}$ -HTX (30  $\mu$ M) the first, fifth and last EPCs were 60, 67 and 70% of control, respectively. Thus, the complete block of peak

amplitude without further shortening of  $\tau$  during activation produced by the agonist in presence of HTX or  $H_{12}$ -HTX suggests that the agent reacts with the channel in resting or closed conformation, open conformation, and at least one intermediate nonconducting stage.

In our pursuit of toxins which will be able to decrease transmitter release and of a definition of their molecular targets and potential use as antidotes for deadly organophosphorus compounds, we have been able to obtain from Dr. John Daly a toxin in very pure form which specifically reacts with sites located on the sarcoplasmic reticulum as well as on the presynaptic nerve terminal. The importance of this toxin's action at the presynaptic nerve terminal is its ability, at low concentrations, to increase transiently the evoked transmitter release and subsequently depress it. In addition, on the postjunctional membrane, although our initial studies revealed that certain analogs of the toxin inhibited the binding of  $\alpha$ -BuTX, the toxin was most effective in inhibiting the reaction of phosphorylated compounds with the ACh receptor and also its ionic channel and, importantly, those organophosphorus compounds that produce an immense effect on the presynaptic nerve terminal such as soman. The toxin, named pumiliotoxin (PTX), is one of a new class of indolizidine alkaloids isolated from the skin secretions of the *Mendrobotid* frogs.

The pumiliotoxins have several derivatives (A, B, C and D), however in this report we will describe only PTX-A and B. PTX-B, although increasing indirect muscle twitch, blocks it subsequently, while PTX-A specifically reacts with the ACh receptor and blocks it partially, having also an effect on the ionic channel (Albuquerque, Daly, and Nimit, unpublished results). Our preliminary results on these two toxins are summarized in the following paragraphs.

PTX-B reversibly potentiates and prolongs the direct elicited muscle twitch in rat and frog skeletal muscle up to 12-fold in a concentration- and frequency-dependent manner, the potentiation being greater at the lower frequencies of stimulation. Responses of the muscle to tetanic stimulation in the presence of PTX-B are potentiated more at 10 and 20 Hz than at 50 and 100 Hz; tetanic fusion occurs earlier and an after-contraction is present when tetanic stimulation occurs in the presence of PTX-B. The twitch/tetanus ratio at 100 Hz is increased in the presence of PTX-B from 0.3 to more than 1.1 as a result of the increase in twitch amplitude. These effects on frog skeletal muscle are seen in the absence of any effects of PTX-B on spontaneous and evoked transmitter release, acetylcholinesterase activity, muscle action potential, delayed rectification and cable properties of the muscle fiber. In the absence of external calcium, PTX-B prolongs but does not potentiate the twitch while methoxyverapamil and dantrolene only partially suppress the actions of PTX-B. In crayfish skeletal muscle, PTX-B increases the rate of rise of the 'calcium-dependent' action potential and shortens its duration. Biochemical studies reveal that PTX-B inhibits calcium-dependent adenosine triphosphatase from sarcoplasmic reticulum preparations of both frog and rat skeletal muscles in a concentration- and calcium-dependent manner (Albuquerque et al., in press). We suggest that PTX-B potentiates and prolongs the muscle twitch: i) by facilitating the release of calcium from storage sites within the sarcoplasmic reticulum; ii) by mobilizing calcium from extracellular sites; and iii) by blocking the reuptake of calcium by calcium-dependent adenosine triphosphatase.



PTX-A and C on the other hand recently have been shown to have actions which are quite different from those of PTX-B. Although our work is still in the early stages, it is clear that PTX-A has both pre- and postsynaptic effects which will be useful to counteract the effects of excess agonist, ACh, at the synaptic cleft as the result of anticholinesterase agents. The presynaptic effects of the toxin appear to decrease transmitter release, while the partial block of the nicotinic ACh receptor and effects on the ionic channel, both in the closed and the open conformation, also tend to inhibit the effects of excessive amounts of the transmitter. Thus it appears that PTX-A and other analogs of PTX which are now in initial stages of investigation may be extremely useful agents to antagonize intoxication by phosphorylated compounds.

Two other agents which have great potential to antagonize the effects of excess agonist are meproadazine and a synthetic agent derived from HTX and now produced in large quantities for our laboratory called desamyl compound.

A number of other nicotinic antagonists are now under investigation in our laboratory, and among them are PCP and its derivatives. Some of these are psychoactive and others are not. The ones that are devoid of hallucinogenic or psychomimetic effects still keep the antinicotinic effect, thus making them ideal antagonists for the excess agonist which is present when cholinesterase is blocked by phosphorylated compounds.

Our description of the PCP compounds begins with the psychoactive, parent compound PCP. The effects of phencyclidine (PCP) were studied on the electrical and chemosensitive properties of the neuromuscular junction (Tsai et al., 1980) and on the EPC, miniature endplate current (MEPC) and synaptic noise of the frog sartorius muscle (Albuquerque et al., 1980a). PCP potentiated both the directly and the indirectly elicited muscle twitch, an effect which occurred with a simultaneous prolongation of the falling phase of the action potential blockade of delayed rectification and only a slight decrease in the rate of rise of spike activity. The prolongation of the action potential was also increased as a function of the frequency of nerve stimulation. In contrast to the marked potentiation of directly elicited muscle twitch, indirect muscle twitch was only transiently potentiated at concentrations lower than 60  $\mu\text{M}$  and subsequently blocked. Indeed, at concentrations higher than 60  $\mu\text{M}$ , blockade of neuromuscular transmission occurred with little or no potentiation of the indirectly elicited twitch (Tsai et al., 1980).

Resting membrane potential and passive electrical properties were little affected by PCP. At high concentrations of PCP the miniature endplate potentials were blocked, as were the ACh sensitivities of the junctional region of innervated muscles as well as the extrajunctional region of chronically denervated muscles. PCP decreased the sensitivity to repetitive microiontophoretic application of ACh. PCP did not prevent the irreversible effects of  $\alpha$ -bungarotoxin on ACh sensitivity in junctional regions of the innervated and extrajunctional regions of chronically denervated muscles. At these effective concentrations (i.e., 1 to 100  $\mu\text{M}$ ) PCP caused negligible inhibition of ACh-esterase. In addition, since PCP did not inhibit the binding of [ $^3\text{H}$ ]ACh or [ $^{125}\text{I}$ ] $\alpha$ -bungarotoxin to the ACh receptors, it was suggested that the inhibition of ACh-receptor-regulated ionic conductances was not due to the inhibition of ACh-receptor binding sites. Inhibition was

possibly due to an interaction with the ionic channel of the ACh receptor. Furthermore, the effect of PCP on the electrical excitability of muscle membrane, shown by the marked prolongation of the action potential and inhibition of delayed rectification, suggested that the agent caused significant blockade of potassium conductance. This effect most likely could account for the potentiation of the muscle twitch.

PCP decreased the peak amplitude of the EPC in the frog sartorius muscle in a voltage- and time-dependent manner, caused nonlinearity in the current-voltage relationship, accelerated the decay time constants of the EPC and MEPC, and shortened the mean lifetime of the single ionic channel (Albuquerque et al., 1980b).

PCP also inhibited binding of [ $^3$ H]H<sub>12</sub>-HTX to the ionic channel of the ACh receptor with a K<sub>d</sub> of 6.9  $\mu$ M. When carbamylcholine was present to activate the ACh receptors, the K<sub>d</sub> value for PCP binding to ionic channel sites was reduced from 10.3  $\pm$  1.3  $\mu$ M to 2.0  $\pm$  1.3  $\mu$ M, thus showing higher affinity for the activated ionic channel sites. In addition, PCP also reacted with the closed ionic channel since a time-dependent effect on EPC amplitude in hyperpolarized membranes was observed even before the ACh receptor was activated. Further PCP depressed peak EPC amplitude more markedly than it shortened the EPC decay time constant, thus disclosing that the depression of the former cannot be accounted for totally by the action of the agent on the open conformation of the ionic channel. A hybrid model was proposed to account for the interactions of PCP with the open and closed states of the ionic channel of the ACh receptor. The actions of PCP on both states of the ionic channel are qualitatively similar to those seen with histrionicotoxin.

Although PCP can antagonize some of the effects of anticholinesterase agents, it is nevertheless a hallucinogenic agent which induces psychomimetic behavior. Indeed, the majority of its derivatives are potentially able to produce such effects. However, recently we have found that although 1-piperidinocyclohexane-carbonitrile (PCC) and *m*-nitro-PCP (N-PCP) are devoid of psychopathological behavioral effects. PCC and N-PCP, in contrast to PCP, are also devoid of any significant effect on potassium conductance, as determined qualitatively by measurement of delayed rectification, and half-decay time of the action potential. Thus the PCP derivatives are providing important clues regarding the mechanisms which form the basis of the alterations in behavioral patterns induced by drugs such as PCP. PCC and N-PCP, devoid as they are of effects on potassium conductance and behavior, retain their effects on the ionic channels of the nicotinic receptor (Albuquerque and Aguayo, unpublished results).

Behaviorally active PCP and *m*-amino-PCP (A-PCP) and inactive N-PCP and PCC were studied on ionic channels of electrically excitable membranes and nicotinic receptors of frog sartorius muscles. The duration and magnitude of twitch potentiation with N-PCP was much less than with PCP or A-PCP. The latter agents produced a frequency-dependent prolongation (300%) of spike duration and blocked delayed rectification. Like PCP (Albuquerque et al., 1980b), N- and A-PCP (30  $\mu$ M) decreased the peak amplitude of EPCs at membrane potentials from +50 to -180 mV and shortened its time constant of decay. The nonlinearity and pattern of voltage sensitivity of EPCs with PCP, N- and A-PCP may result from the drugs' interaction with a site sensitive to the potential field across the junctional membrane. The agents appear to bind

to several sites in the ionic channel; in its various conformational states. The behavioral changes elicited by PCP and A-PCP may be related to a decreased K conductance. Thus, the apparent lack of effect of N-PCP and PCC on K conductance and the persistence of their nicotinic effect may account for the absence of abnormal behavior patterns. It is clear, therefore, that these analogs of PCP which are devoid of behavioral effects may be extremely useful agents to counteract the lethal effects of phosphorylated compounds. Indeed, N-PCP is sufficiently powerful to allow experimental animals to tolerate intoxication with anticholinesterase agents such as DFP and neostigmine and survive upon the administration of the agent (Albuquerque et al., unpublished results).

Similar to atropine the muscarinic antagonist quiniclidinyl benzilate (BZ) could be of potential use as an agent to antagonize the actions of certain organophosphate compounds on the muscle as well as the nicotinic receptor. However, because BZ reacts with the ionic channel of the nicotinic receptor and in much larger concentrations than at the muscarinic receptor (see next paragraph), it is of negligible value as an agent to antagonize some of the nicotinic effects produced by excess of the agonist.

Electrophysiological studies (Schofield, et al., 1981a) using conventional microelectrode technique revealed that BZ ( $5$  to  $50 \times 10^{-6}$  M) reacts with sites sensitive to the agent at both the pre- and post-junctional regions of the nicotinic synapse, including the open conformation of the ionic channel associated with the nicotinic ACh receptor. BZ decreased the endplate potential (EPP) and miniature endplate potential amplitude while decreasing the quantal content of the EPPs. The agent increased the latency of the EPP, EPC and nerve terminal spike in a frequency-dependent manner. Since conduction velocity in the sciatic nerve and synaptic delay were unaffected, the result indicated that the site of action of the agent is the unmyelinated nerve terminal. BZ decreased the rate of rise and amplitude of the muscle action potentials and increased the half-decay time. Increasing the stimulation frequency potentiated these effects. These results are consistent with a blockade of the electrically excitable sodium channels. BZ decreased the amplitude and time constant of decay of the EPCs (Fig. 9) and miniature EPCs as well as the lifetime of the single ACh-activated ionic channels, thus revealing an action of the agent on the open conformation of the ionic channel of the nicotinic ACh receptor.

An important aspect of our electrophysiological investigations into the pharmacology of the nicotinic ionic channel has been the identification for the very first time of sites on the outside and inside of the cell membrane which regulate the behavior of the channel (Aguayo et al., 1981, in press). The actions of several quaternary molecules, which unlike their tertiary analogs, are very polar and will not penetrate lipid phases or cross membranes readily on the endplate region of the frog sartorius muscles were studied using bath application or intracellular injection. Tetraethylammonium (TEA), atropine methyl bromide, phencyclidine methiodide (PCP methiodide), piperocaine methiodide and N-methylpiperidine methiodide were injected into the sarcoplasm just beneath the post-junctional membrane and 250-350  $\mu$ m away from the endplate region. The ability of these agents to depress K conductance and prolong the muscle action potential was used as a measure of the efficacy of intracellular drug administration. External application of TEA (50-1000  $\mu$ M) decreased the peak amplitude of the EPC and its time constant

of decay ( $\tau_{EPC}$ ), but this agent and atropine methyl bromide were ineffective when injected internally. PCP methiodide (3-30  $\mu M$ ) and piperocaine methiodide (10-60  $\mu M$ ) had a potent action on EPCs and spontaneous miniature endplate currents when applied to either side of the membrane. Both agents caused nonlinearity of the peak amplitude and a shortened lifetime in spite of the fact that they sense only 6% of the membrane potential at their binding sites. Internal application of PCP methiodide and piperocaine methiodide caused significant depression of the EPC and MEPC peak amplitude and simultaneous shortening of the decay time constant.

The decay time constant of the EPC and MEPC in the presence of PCP methiodide and piperocaine methiodide was shorter at less negative potentials (i.e., -60 mV) than at more negative membrane potentials (i.e., -100 mV). Similar results were obtained with internal application of piperocaine methiodide. The ability of PCP methiodide and piperocaine methiodide to block the ionic channel of the acetylcholine receptor both from outside and inside the membrane, in contrast to TEA and atropine methyl bromide shows that the active sites for drug binding may explain some of the mechanisms related to the voltage and time-dependent effects of agents and a possible asymmetry of the ionic channel.

Among the many agents whose actions at the nicotinic receptor-ionic channel complex we have elucidated during the course of this project, is a very interesting nicotinic agonist Anatoxin-A. Anatoxin-A is an exotoxin isolated from a filamentous, freshwater blue-green alga, Anabaena flos-aquae. Windblown concentrations of the algae surface blooms have killed livestock and waterfowl that ingest the water die rapidly. The cause of death is respiratory paralysis, and the mode of action has the characteristics of a depolarizing neuromuscular blocking agent. We have completed detailed studies of the action of the toxin at the frog neuromuscular junction (Spivak et al., 1980) including voltage clamp studies of the EPC and MEPCs and noise analysis. Anatoxin-a specifically reacted with the ACh receptor, and was devoid of a significant effect on the ionic channel. Anatoxin-a caused depolarization of the junctional membrane, neuromuscular blockade, contracture of the frog's rectus abdominis muscle, desensitization and alteration of the action potential. It produced steady endplate currents that yielded estimates of mean channel lifetime and channel conductance very similar to those of ACh. In comparison with other agonists reported in the literature, e.g., decamethonium, succinylcholine, carbamylcholine and acetylcholine, anatoxin-a was found to be the most potent.

Previous studies in our laboratory have demonstrated that high doses of carbamates can induce severe alterations of behavior in animals which culminate in a generalized state of weakness. In the rat we have been able to observe that neostigmine causes at high concentrations marked degeneration of the postsynaptic region of the ACh receptor (Tiedt et al., 1978), with, in addition, an effect on the ACh receptor and its ionic channels. This effect, however, is much less evident than that produced by the agents which block cholinesterase. During the last year of the grant we have been able to study several agents which antagonize the effects of neostigmine on the ACh receptor and ionic channel and also decrease the effects of an excess of ACh. As described earlier in the report, the agents of primary choice are PTX-A and m-nitro-PCP (Albuquerque et al., unpublished results).

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A major thrust of our studies has been the use of several tricyclic antidepressants, imipramine (IMIP), desimipramine, amitriptyline, nortriptyline and protriptyline. These agents, however, react with the channel in quite dissimilar manners. Our preliminary results indicate that these tricyclic antidepressants react with different sites within the ionic channel. We are able to clearly separate the binding sites along the channel responsible for voltage-dependence, voltage and time dependence, and shortening of the decay time constant. The channel thus is a molecular target that can have any number of different binding sites and is able to generate levels of energy barriers which are a direct function of the conformational change allowed by the effects of a given agent, in this case, the tricyclic antidepressants (Schofield et al., 1981). For the sake of simplicity, we will limit our discussion to imipramine.

The effects of imipramine on ionic channels of nicotinic receptors and electrically excitable membranes were studied on the frog sartorius muscle (Schofield, et al., 1981; Eldefrawi et al., 1981). IMIP (5-10  $\mu$ M) depressed the amplitude and rate of rise of directly-evoked action potentials elicited singly and in a frequency-dependent manner without altering the half-decay time of the spike. This, and the lack of IMIP's effect on delayed rectification suggest a partial suppression of Na conductance. The peak amplitude of endplate currents (EPCs) at negative potentials were also depressed by IMIP in a concentration- and time-dependent manner. A significant departure from the linear current-voltage relationship occurred with IMIP, only slightly affecting the time constant of EPC decay). Fluctuation analysis disclosed a small but significant increase in channel conductance and a decrease in channel lifetime with IMIP. The drug induced rundown of repetitively-evoked ACh potentials, in a frequency- and duration-dependent manner. IMIP did not prevent the reaction of  $\alpha$ -bungarotoxin with the ACh receptor. The greater depression of EPC vs. miniature EPC amplitude by IMIP may reflect a decreased quantal release of ACh. Thus, IMIP may interact with the activated, nonconducting species of the ionic channel; and may also cause a partial block of the channel in its open conformation.

The effect of nereistoxin (NTX) was studied, by electrophysiological methods in neuromuscular transmission in frog sartorius and rat diaphragm muscles. NTX blocked the indirectly elicited twitch tension but not the directly elicited ones, and did not affect action potential, quantal content and frequency of the spontaneous MEPP. The postsynaptic inhibition by NTX was evident from the reduction it caused in the amplitudes of the endplate potential and endplate current as well as the extrajunctional ACh sensitivity of denervated rat soleus muscle, and its inhibition of binding of [ $^3$ H]ACh and [ $^{125}$ I]  $\alpha$ -bungarotoxin to Torpedo ACh receptors. In addition, NTX caused initial postsynaptic depolarization and potentiation of the indirectly elicited twitch tension. Since NTX did not inhibit binding of [ $^3$ H]perhydrohistrionicotoxin and did not alter the linearity of the current voltage relationship, nor the time course of endplate current in frog sartorius muscle, we suggested that its inhibition of neuromuscular transmission was due to its inhibition of the ACh-receptor sites and not the ionic channel sites. Also, NTX acted as a partial agonist since it could activate the ACh receptor, although its major action was that of an antagonist. In conclusion, NTX blocks neuromuscular transmission without affecting presynaptic events or inhibiting ACh-esterase activity. Its molecular target is the ACh receptor with which it interacts in a dual

manner. Although NTX activates the ACh receptor slightly, its major effect is inhibition of the receptor action without affecting the ionic channel sites. Both biophysical and biochemical data suggest that NTX acts as a partial agonist or antagonist of the ACh receptor.

The studies using radioactive batrachotoxin (BTX) are of particular relevance because we found earlier that BTX, although not reacting with the ACh receptor-ionic channel complex, was able to depress the depolarization induced by carbamylcholine (Garrison, et al., 1978) (Fig. 14). This depolarization has now been found to be occurring in the coupling system between the ionic channel of the ACh receptor and the lipid moiety of the electrically excitable membrane. The use of the radioactive batrachotoxin-A 20  $\alpha$ -benzoate would enable us to evaluate again the molecular events involved in the action of the toxin. The paragraphs below illustrate our general results using the toxin.

Batrachotoxinin-A 20- $\alpha$ -benzoate (BTX-B), an analog of the potent depolarizing agent batrachotoxin (BTX), was prepared by selective esterification of naturally-occurring batrachotoxinin-A with benzoic acid. Use of  $p$ -[ $^3$ H]benzoic acid yielded a labeled derivative with a specific activity of 18 Ci/mmol and > 90% radiochemical purity.

BTX-B depolarizes rat phrenic nerve-diaphragm preparations with a time course and concentration dependence virtually indistinguishable from that of BTX. Depolarization with either toxin leads to i) muscle contracture having two temporally distinct phases, ii) blockade of the contracture-inducing effects of both isotonic KCl and caffeine, iii) blockade of indirectly-evoked twitch followed by blockade of directly-evoked twitch and iv) an initial precipitous increase in the frequency of spontaneous miniature endplate potentials which subsequently falls to zero. The electrophysiological effects of BTX-B, as with BTX are not reversed by washing BTX are blocked by tetrodotoxin.

Equilibrium binding of [ $^3$ H]BTX-B to mouse cerebral cortex homogenates was investigated using a pellet assay procedure. A specific, saturable component of binding was measured, described by an equilibrium dissociation constant of 0.7  $\mu$ M and a maximum number of binding sites of 90 pmol per gram of tissue (wet weight). Specific binding is inhibited by BTX and other BTX analogs, veratradine and grayanotoxin but is unaffected by tetrodotoxin and cevine. Under conditions of this assay, neither crude Leiurus quinquestriatus scorpion venom nor purified sea anemone toxin II has any effect on specific binding.

The data support the conclusion that BTX-B interacts with a recognition site associated with voltage sensitive sodium channels which is identical to the recognition site for BTX.

The interaction of naltrexone, morphine (+ and -) and enkephalins, levallorphan with the ionic channels at synapses of the frog sartorius muscle has been investigated with conventional techniques for measurement of endplate or miniature endplate currents (EPC and MEPC) and acetylcholine noise analysis. In this report we shall describe only the effect of the so-called pure narcotic antagonist, naltrexone. Naltrexone, a pure opiate antagonist, shortens the time constant of decay ( $\tau$ ) of both EPC and MEPC. A semilogarithmic plot of  $\tau$  vs. membrane potential reveals a curvilinear

relationship prominent at negative to positive potentials whose onset of inflection seen at negative potentials is a function of the drug concentration. At 60 and 300  $\mu\text{M}$ , naltrexone accelerated the single exponential decay of the EPC, at -150 mV, from 3.3 to 0.8 msec and from 3.3 to 0.3 msec, respectively. The pattern shows a nonlinear function when the inverse of  $\tau$  was plotted against the drug concentration at the hyperpolarized region. At drug concentrations of 300  $\mu\text{M}$ , EPC peak amplitude was decreased to values less than 10% of the corresponding control condition. The onset of the nonlinearity in the current/voltage relationship was also dependent on the drug concentration. The ability of naltrexone to interact with the open conformation of the ionic channel was further evidenced by an increase in drug efficacy at a lower temperature (i.e., 10° C). Acetylcholine noise analysis experiments show that channel lifetime and single channel conductance parameters also decreased in a voltage dependent manner and as a function of drug concentration. At a membrane potential of -75 mV and drug concentration of 40  $\mu\text{M}$ , the values for channel lifetime and single channel conductance were 0.6 msec and 16 pS, respectively, vs. 1.2 msec and 26 pS for control conditions. Further investigations on naltrexone suggest that the action of this agent is on the closed and open conformations of the channel. Levallorphan, a mixed antagonist of morphine also reveals a similar depressant action on both the peak amplitude and  $\tau$  of the EPCs although some striking differences were noticed. Levallorphan has a stronger depressant effect on EPC amplitude than naltrexone. Indeed, levallorphan (100  $\mu\text{M}$ ) markedly depressed the peak amplitude of the EPC such that at -150 mV, the peak amplitude of the EPC was reduced to about 10% of control values and such an effect was highly voltage dependent. The ability of these agents to react with ionic channels of the acetylcholine receptor at concentrations similar to those used for opiate antagonism suggests that in addition to reacting with the opiate receptor, these agents display similar potencies at other types of receptor-ionic channel complexes.

#### 5b.iii. Conclusion

Electrophysiological techniques were used to analyze the characteristics of endplate currents and channel properties of mammalian and frog skeletal muscles, while biochemical techniques were used to study the molecular characteristics and drug specificities of the ACh-receptor/channel of the electric organ of the electric ray, Torpedo sp.

The data obtained suggest that the ACh-receptor/channel molecule carries two kinds of binding sites: receptor sites that bind ACh and  $\alpha$ -bungarotoxin in a voltage-independent and almost temperature-independent manner, and channel sites that bind a variety of drugs in a voltage- and temperature-dependent manner, and are more sensitive than the receptor sites to treatment by the detergent Triton X-100 and sulfhydryl reducing and alkylating agents. The drugs that bind to the channel sites include perhydrohistrionicotoxin, the antiviral and antiparkinson drug amantadine, local anesthetics (e.g., piperocaine), opiate antagonists (e.g., naltrexone) and agonists (e.g., levallorphan and cyclazocine), the antimalarial quinacrine, antibiotics (e.g., polymyxin, gentamicin and aminoglycosides), tricyclic antidepressants such as imipramine, the general anesthetic and hallucinogen phencyclidine, as well as the receptor site antagonists decamethonium and curare, anticholinesterase quaternary ammonium compounds and aliphatic alcohols. The molecular diversity of these drugs demonstrates the relative nonspecificity of the channel sites,

while the high affinities that many drugs have ( $K_d < 1 \mu M$ ) suggest that they may be secondary targets for many drugs. Two of the toxins studied (anatoxin and nereistoxin) did not bind to the channel sites, but rather to the receptor sites, acting as agonists and antagonists, respectively. There are different ionic channel sites reachable either from only the outside of the membrane (e.g., atropine methyl bromide and tetraethylammonium) or from both outside and inside (e.g., PCP methiodide and piperocaine methiodide). In addition, PCP and perhydrohistrionicotoxin bind competitively to the same channel sites, while imipramine appears to bind noncompetitively to different and fewer number of channel sites.

The ACh-receptor/channel molecule undergoes different ligand-induced conformations, which we could detect by monitoring the apparent rates of binding of radiolabeled drugs to the channel sites. These are the resting, agonist-induced, antagonist-induced and desensitized conformations. Several of the channel drugs bind to open as well as closed channel conformations, and some such as imipramine bind preferentially to an intermediate activated but nonconducting conformation. The allosteric effect of receptor site occupation on channel site affinity is inhibited by  $Ca^{2+}$ , while  $F^-$  reverses the  $Ca^{2+}$  effect.

It is evident that the pharmacology of the ionic channel sites of the nicotinic ACh-receptor is very different from, and less specific than, that of the receptor sites. The high affinity the channel sites have for many drugs make them viable, though secondary, targets for their actions. This has importance particularly in compromised neuromuscular transmission such as in certain patients (e.g., with myasthenia gravis) and in cases of poisoning by anticholinesterases.

#### 5c. List of Publications

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5d. List of Participating Scientific Personnel Showing Advanced Degrees Earned While Employed on the Project

No one earned advanced degrees while employed on the project.

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